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RESEARCH ARTICLE

# Effect of the prenatal electromagnetic field exposure on cochlear nucleus neurons and oligodendrocytes in rats

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## Abstract

Electromagnetic radiation from elecromagnetic field (EMF) sources has been an important health concern for a long time. The vast majority of this exposure is due to the widespread use of mobile phones, an important source of the EMF. The EMF generated by mobile phones may have adverse effects on the various biological structures that regulate the body system and function. In this study, it was aimed to evaluate histopathologically the effects of 900-megahertz (MHz) EMF application in the prenatal period on the development of the ventral cochlear nucleus, which is the first place of hearing in the brainstem, at various time points of the postnatal period in rats. In the study, Sprague–Dawley pregnant rats were divided randomly into two groups as the control group and the EMF group. The rats in the EMF group were exposed to a 900-MHz EMF every day until birth, while no EMF was applied to the rats in the control group. Auditory brainstem responses of both groups were recorded on the postnatal 13th day, the day the hearing starts. Newborn rats were sacrificed by anesthesia on days 7, 10, 15, and 30. Contrary to the control group, structural damage in cochlear nuclear neurons and oligodendrocyte cell structures and increased caspase-3 activity were observed in the postnatal period in the EMF groups. However, no significant difference was observed between the groups in terms of structural damage and caspase-3 activity at different stages of the postnatal period when cochlear nucleus development was observed. According to ABS, there was no significant difference between the average latency of waves in both groups. In conclusion, this study shows that 900-MHz electromagnetic waves propagated from mobile phones during the prenatal period have no harmful effects on the development of the ventral cochlear nucleus of rats.

**Keywords** Cochlear nucleus · Electromagnetic field · Prenatal · Postnatal · Rat

## Introduction

The electromagnetic field (EMF) is an important environmental agent affecting living organisms (Deruelle 2020). More than three billion people daily in more than 200 countries are consciously exposed to EMF (Fragopoulou et al. 2010). Mobile phones with widespread use constitute a large part of EMF exposure. While there were almost 500 million mobile phone users worldwide at the end of 2000, this number exceeded 7 billion at the end of 2015 (Sanou 2015). As mobile phones communicate by emitting radio signals, the widespread use of mobiles causes people to be exposed to EMF at various frequencies. The EMF frequency emitted by mobile phones varies between 800 and 2000 MHz, which are categorized as radiofrequency waves. According to the Global Mobile Communication System, 900 MHZ is one of the most widely used frequency bands (Yahyazadeh and Altunkaynak 2019; Wallace et al. 2020). The increasing prevalence of mobile phone use raises concerns about the impact of the electromagnetic field emitted by these devices on human health.

Radiofrequency EMF associated with mobile phones appear to have deleterious health effects (Vanderstraeten and Verschaeve 2020). Prenatal and postnatal exposure

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to 900-MHz EMF has been reported to potentially cause various disturbances in the physiology, morphology, and behavior of many animal species (İkinci et al. 2013; Odaci et al. 2013; Azimzadeh and Jelodar 2020). In addition to these effects, EMF exposure may affect cell morphology and physiology in various aspects (Zymantiene et al. 2020). Scientific research has shown that EMF has various biological effects even at very low density and strength (Kim et al. 2019). It is claimed that EMF may also have a negative effect on structures that have not yet completed their development (Kaplan et al. 2016).

The cochlear nucleus (CN) is located on the border between pons and medulla oblongata and the VIII synaptic terminal area from the nerve is the first neural center. CN, which contains different morphological and physiological types of neurons, consists of two sub-nuclei: the ventral cochlear nucleus (VCN) and dorsal cochlear nucleus (DCN) (Moore and Linthicum 2007). As it is the first auditory input processing nucleus of the central nervous system, the CN is directly involved in language acquisition and learning, and therefore, it has been the subject of numerous studies in clinical and basic science studies. The rats have an immature hearing system at birth (Geal-Dor et al. 1993). Physiological hearing in rats begins approximately 2 weeks after birth. Studies show that the external ear canals are not opened before the 12th day postpartum (P) and the first auditory brainstem responses are recorded on P 12–P 14 days (Friauf and Kandler 1993). Besides, animal studies with radiographic marking have shown that neurogenesis in CN also occurs within a week before birth (Pierce 1967; Altman and Bayer 1980; Martin and Rickets 1981).

In this study, it was aimed to investigate the effects of 900-MHz EMF application in the prenatal period in rats for the first time in the literature on the development of the VCN, which is the first place of hearing in the brainstem, neurons, and oligodendrocytes in different stages of the postnatal period.

## Materials and methods

### Animals

Four Sprague–Dawley pregnant rats ranging in weight from 280 to 300 g were used in the study. During the study, the rats were kept in the experimental animal unit provided with optimum conditions (12-h (h) light, 12-h dark, 55–60% moisture, and 22 ± 3 °C). The rats were allowed to consume unlimited food and tap water. The study protocol was prepared according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the necessary permissions were obtained from the Local Ethics Committee of Animal Experiments of the University (no: 37/2010). After the pregnancy was followed daily, the rat with copulation plug in the vagina was separated as pregnant and the day the plug was observed was accepted as the first day of pregnancy (Munoz-Perez et al. 2020).

### Experimental design

Pregnant rats were randomly divided into two groups of 6 each as control and EMF groups. Pregnant rats in the EMF group were exposed to a 900-MHz magnetic field from the antenna placed under the cage (5 cm away) for 1 h a day during pregnancy (Bedir et al. 2018) (Tumkaya et al. 2019, Ertilav et al. 2018). Electromagnetic waves were generated using an EMF generator (Anritsu MG3670B, Kanagawa, Japan). The generator produced a signal at a level of 5.4 dBm (3.47mW), at 900 MHz at the mobile phone talk mode. The SAR (specific absorption rate) value of 0.087 W/kg was calculated according to the study of Qin et al. (2012). Pregnant animals in the control group were not exposed to EMF. In our study, 6 pregnant animals in each group were used in the experiment. Eight to 12 puppies were born from each pregnant rat. Six of the newborn were randomly selected and allocated into four groups as postnatal 7th (P7), 10th (P10), 15th (P15), and 30th (P30). Newborn rats were taken from postnatal control and EMF groups, which were allocated into four groups as P7, P10, P15, and P30 ($n = 6$). New-born rats were cared for during the experiment in an environment that provided the optimum conditions mentioned above. Previous morphological studies showed that in rodents, auditory function emerges 12–14 days postpartum. In the present work, we studied the aforementioned days after birth. The subjects were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar ®, Parke-Davis, Eczacibasi, Istanbul, Turkey) and 10 mg/kg Xylazine HCl (Alfazyne ®, Alfasan International B.V. Woerden, Netherlands) on P7, P10, P15, and P30 days and the animals were sacrificed. The VCN of rats was removed for histopathological examinations.

### Auditory brainstem response measurement

The auditory brainstem response (ABR) test is used to determine a child's hearing ability. This measures the response to different sounds by cochlear and brain neurons (Özgür et al. 2015). Measurements of ABR were recorded under anesthesia on the P13th day. Otoscopic examination of all rats was performed to exclude factors that could affect test results before measurement. Subcutaneous needle electrodes were used for the test (Özgür et al. 2015). We performed ABR recordings at 30, 50, and 70 dB nHL click stimuli, using 0.3–3.0 kHz filters and based on the 500 record average. We analyzed recordings at 50 dB nHL, since the best wave morphology was obtained at this intensity.

### Histopathological analysis

For histopathological examinations, the VCN taken from rats was fixed in 10% neutral formalin solution. Following routine follow-up procedures, 4-µm sections were taken from the CN tissues embedded in paraffin. These were stained with hematoxylin–eosin (H-E) and examined under a light microscope (BX51, Olympus, Tokyo, Japan) and photographed with a DP 20 camera (Olympus, Tokyo, Japan).

### Immunohistochemical analysis

For immunohistochemical examinations, VCN sections were placed on positive charged slides. The sections were deparaffinized with xylol (Merck) and dehydrated by passing through increasing alcohol (Merck) series. They were stained immunohistochemically using a commercial kit with secondary antibody (HRP/DAB–ABC-Detection IHC Kit, ab6205718, Abcam, UK) to examine caspase-3 activity in the tissue after dehydration. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide (ab64261, Abcam) treatment for 10 min. After washing with PBS, sections were incubated with serum blocking solution (ab64261, Abcam), and proteinase K in PBS for the antigen retrieval stage was added (20 µg/mL) (ab64220, Abcam) on the sections. Sections immersed in equilibration buffer (ab64261, Abcam) were incubated for 60 min with anti-active caspase-3 antibody (rabbit polyclonal, ab2302, Abcam, UK) following the manufacturer's dilution recommendation. Tissues were then treated with streptavidin HRP (ab64261, Abcam) and sections were stained with DAB (ab64261, Abcam) after washing with PBS. For contrast staining, slides were stained with Mayer's hematoxylin before covering with a coverslip, then examined under a light microscope and photographed with a DP20 camera.

### Semi-quantitative analysis

VCN sections stained with caspase-3 were evaluated under a light microscope (BX51, Olympus, Tokyo) at × 40 magnification. Ten non-overlapping areas randomly selected for each CN section were evaluated blindly by two independent histopathologists. Caspase-3 positivity was scored as weak (≤ 5%, grade 1), moderate (≤ 25%, grade 2), intense (≤ 50%, grade 3), and very intense (≤ 75%, grade 4) (Mercantepe 2018).

### Statistical analysis

Data obtained as a result of half-quantitative analysis were calculated using SPSS 20.0 (IBM Corp., IL, USA) statistical program. The results were expressed as median and interquartile range (25–75%). The data were analyzed using the Tamhane T2 test following the Kruskal–Wallis test. $P$-value < 0.05 was chosen as significant.

## Results

### ABR results

No significant difference was observed in the wave latencies between both of the ears of the rats in the records of the ABR.

### Histopathological findings

Light microscopic examinations were performed after H-E staining; VCN structures showed normal histological appearance in the control groups on each investigated day (Fig. 1A–D, Fig. 2A–D). In the EMF groups, hypertrophied necrotic neurons and oligodendrocytes with large vacuoles in their cytoplasm were observed on the test days. Also, shrunken apoptotic neurons and oligodendrocytes with fragmented nuclei were detected in these groups (Fig. 1E–H, Fig. 2E–H).

### Immunohistochemical findings

No immunopositivity was detected in the neurons and oligodendrocytes following caspase-3 immunohistochemical staining of VCN sections of the control groups of each test day (Fig. 3A–B, Fig. 4 A–B). Caspase-3 expression was observed at varying intensities in neurons, especially in oligodendrocytes, in the EMF groups on every test day (Fig. 3C–D, Fig. 4C–D).

### Statistical findings

Cleaved caspase-3 expression of varying intensities was observed in neurons, mainly oligodendrocytes, in the P7, P10, P15, and P30 EMF groups compared to the corresponding control groups ($p < 0.05$) (Fig. 3C–D, Fig. 4C–D) (Table 1). Although the positivity of caspase-3 in the P10 EMF group neurons and oligodendrocytes increased compared to the P7 EMF group, the difference was insignificant ($p > 0.05$) (Fig. 3C–D, Fig. 4C–D) (Table 2). Similarly, even though caspase-3 positivity was higher in the P15 EMF vs the P10 EMF group and the P30 vs P15 EMF group, the differences were not significant ($p > 0.05$) (Fig. 3C–D, Fig. 4C–D) (Table 2). Overall, we found that there was an increase in the expression of caspase-3 in the EMF groups compared to the control groups. When EMF groups were evaluated among themselves, there was no significant difference in caspase-3-positive neuron and oligodendrocytes ($p > 0.05$).

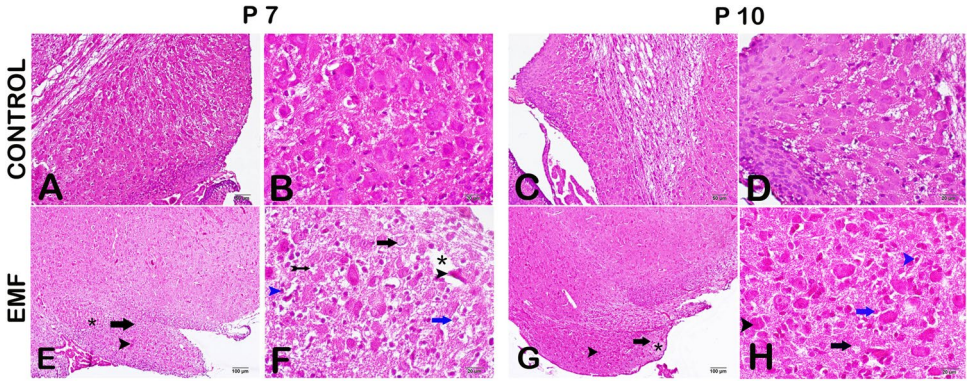


**Fig. 1** Light microscopic view of sections of VCN stained with H-E. **A** (×20)–**B** (×40) P7 control group; image of healthy VCN tissue belonging to the P7 control group. **C** (×20)–**D** (×40) P10 control group; image of healthy VCN tissue belonging to the P10 control group. **E** (×20)–**F** (×40) P7 EMF group; image of damaged VCN tissue belonging to the P7 EMF group. It is observed that neurons decrease in number and are replaced by large edematous areas (asterisks). Shrunken apoptotic neurons (blue arrow) and oligodendrocytes (blue arrowhead) with fragmented nuclei are observed. Hypertrophic neurons (arrow) and oligodendrocytes (arrowhead) that have lost their cytoplasm content and connective complexes are observed. **G** (×10)–**H** (×40) P10 EMF group; image of damaged VCN tissue belonging to the P10 EMF group. It is observed that neurons decrease in number and are replaced by large edematous areas (asterisks). Shrunken apoptotic neurons (blue arrow) and oligodendrocytes (blue arrowhead) with fragmented nuclei are observed. Necrotic neurons (arrow) and oligodendrocytes (arrowhead) with large vacuoles and absent connecting complexes are observed

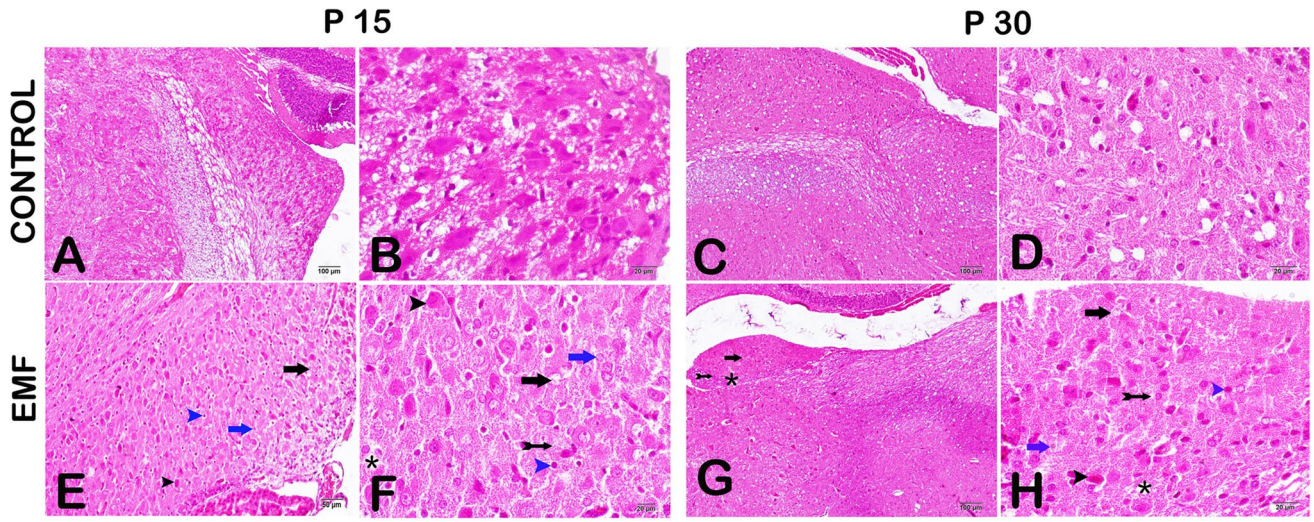


**Fig. 2** Light microscopic view of sections of VCN tissue stained with H-E. **A** (×20)–**B** (×40) P15 control group; image of healthy VCN tissue belonging to the P15 control group. **C** (×20)–**D** (×40) P30 control group; image of VCN tissue in a normal structure belonging to the P30 control group. **E** (×200)–**F** (×40) P15 EMF group; image of damaged VCN tissue belonging to the P15 EMF group. It is observed that neurons decrease in number and are replaced by large edematous areas (asterisks). Shrunken apoptotic neurons (blue arrow) and oligodendrocytes (blue arrowhead) with fragmented nuclei are observed. Hypertrophic neurons (arrow) and oligodendrocytes (arrowhead) that have lost their cytoplasm content and connective complexes are observed. **G** (×10)–**H** (×40) P30 EMF group; image of damaged VCN tissue belonging to the P30 EMF group. It is observed that neurons decrease in number and are replaced by large edematous areas (asterisks). Shrunken apoptotic neurons (blue arrow) and oligodendrocytes (blue arrowhead) with fragmented nuclei are observed. Necrotic neurons (arrow) and oligodendrocytes (arrowhead) with large vacuoles and losing their connecting complexes are observed

## Discussion

In this study, the histopathological effects of 900-MHz EMF on the development of the VCN in rats were investigated for the first time. This study revealed that 1 h of 900-MHz EMF application per day during pregnancy does not affect neurons and oligodendrocytes in the development of the VCN.

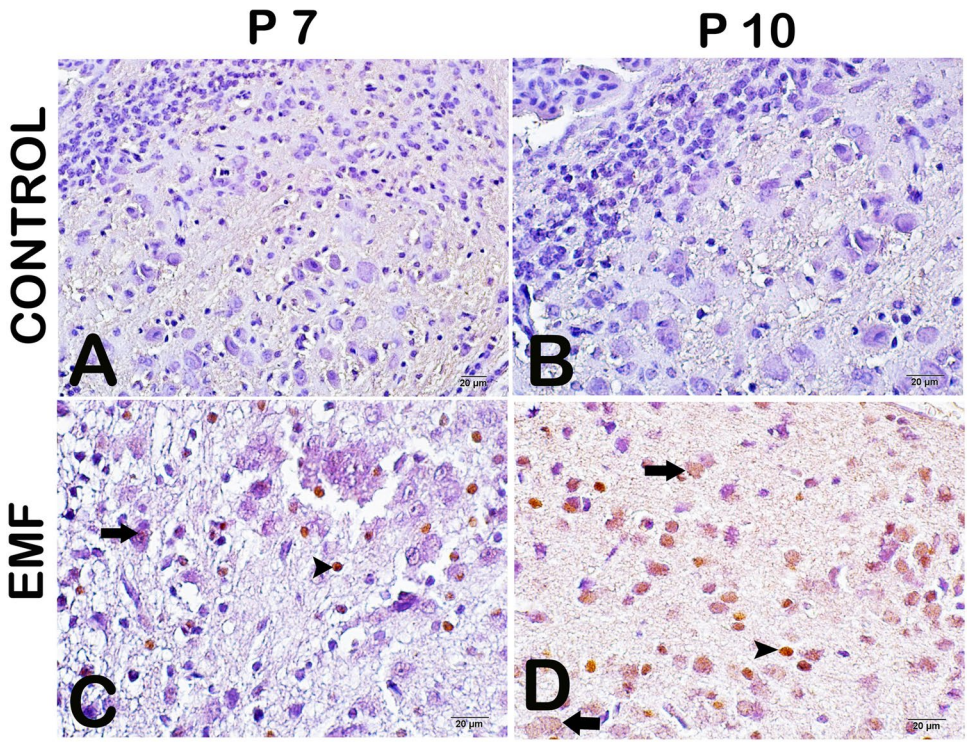


**Fig. 3** Light microscopic view of sections of VCN tissue stained with caspase-3. **A** (×40) P7 control group; caspase-3-negative VCN tissue image of the P7 control group. **B** (×40) P10 control group; image of VCN tissue in normal structure belonging to the P10 control group. **C** (×40) P7 EMF group; moderately positive caspase-3 is observed in neurons (arrow) and oligodendrocytes (arrow). **D** (×40) P10 EMF group; it is observed that the neurons (arrow) and the oligodendrocytes are densely caspase-3 positive (arrow)

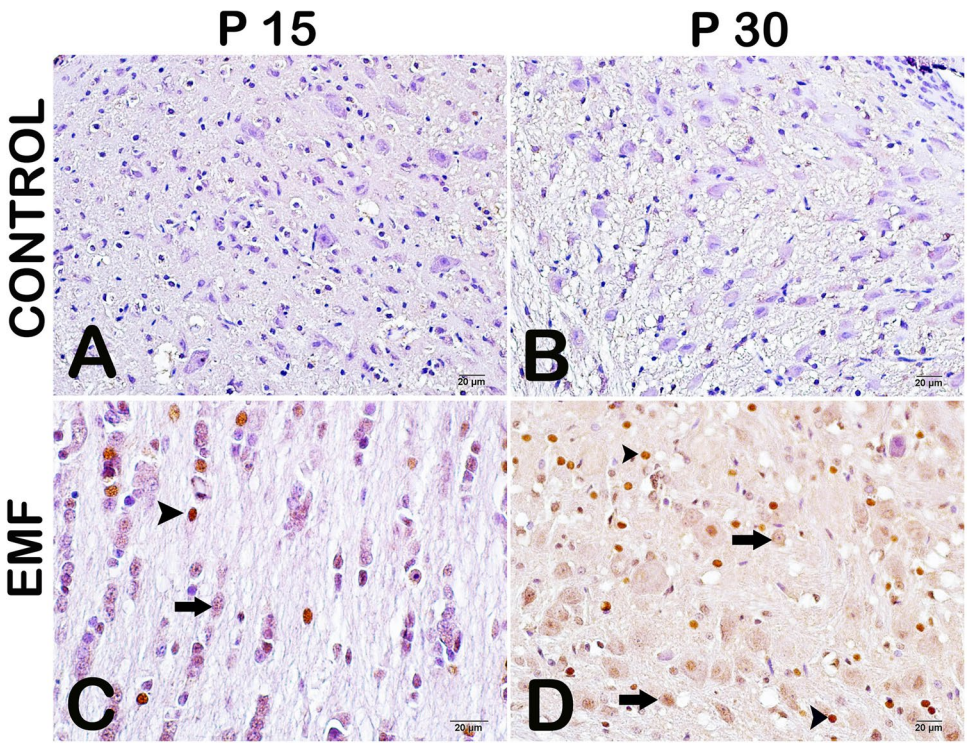


**Fig. 4** Light microscopic view of sections of VCN tissue stained with caspase-3. **A** (×40) P15 control group; caspase-3-negative VCN tissue image of 15 days control group. **B** (×40) P30 control group; image of VCN tissue in a normal structure belonging to the P30 control group. **C** (×40) P15 EMF group; moderately positive caspase-3 is observed in neurons (arrow) and oligodendrocytes (arrow). **D** (×40) P30 EMF group; it is observed that the neurons (arrow) and the oligodendrocytes are dense caspase-3 positive (arrow)

Mobile phones, which are widely used today, constitute an important source of EMF. Advances in technology extend mobile usage and duration (Ulubay et al. 2015). The effects of EMF vary depending on the frequency and intensity of the magnetic field generated by the device including mobiles. For example, televisions have an EMF frequency of 54–700 MHz, microwaves 2.4 GHz, and mobile phones 900 MHz–2.4 GHz. According to the global mobile communication system, 900 MHZ is one of the most widely used frequency bands (Koyu et al. 2005; Panagopoulos

**Table 1** Cleaved caspase-3 positivity score of EMF and control groups

| Groups | Neurons (median ± SD) | Oligodendrocytes (median ± SD) |
|---|---|---|
| P7 control | $0.00 \pm 0.52^{b}$ | $0.00 \pm 0.41^{b}$ |
| P7 EMF | $2.00 \pm 0.41^{a}$ | $2.00 \pm 0.52^{a}$ |
| P10 control | $0.00 \pm 0.52^{d}$ | $0.00 \pm 0.52^{d}$ |
| P10 EMF | $2.00 \pm 0.75^{c}$ | $2.00 \pm 0.41^{c}$ |
| P15 control | $0.00 \pm 0.52^{f}$ | $0.00 \pm 0.41^{f}$ |
| P15 EMF | $2.00 \pm 0.75^{e}$ | $2.00 \pm 0.52^{e}$ |
| P30 control | $1.00 \pm 0.63^{h}$ | $0.00 \pm 0.41^{h}$ |
| P30 EMF | $4.00 \pm 0.52^{g}$ | $4.00 \pm 0.52^{g}$ |

[a] $P < 0.05$: compared to the P7 control group
[b] $P < 0.05$: compared to the P7 EMF group
[c] $P < 0.05$: compared to the P10 control group
[d] $P < 0.05$: compared to the P10 EMF group
[e] $P < 0.05$: compared to the P15 control group
[f] $P < 0.05$: compared to the P15 EMF group
[g] $P < 0.05$: compared to the P30 control group
[h] $P < 0.05$: compared to the P30 EMF group, Mann–Whitney *U* test

et al. 2007). In line with this information, EMF with a frequency of 900 MHz, which meets the standards of the World Communication System and the EMF frequency of mobile phones used in our daily life, was applied to prenatal rats in this study (Topal et al. 2015; Odacı et al. 2016; Türedi et al. 2016).

Recent studies show that EMF exposures at different frequencies have adverse health effects (Miah and Kamat 2017). The effects of 900-MHz EMF on various tissues have been shown in previous studies (Mercantepe 2018; Azimzadeh and Jelodar 2020). Besides, it has been reported in studies that EMF exposure in the postpartum period and the antenatal period causes damage in various tissues, adversely affecting hippocampus tissue and passive avoidance learning and memory (PALM) performance (Ulubay et al. 2015; Türedi et al. 2016; Azimzadeh and Jelodar 2020). Recent studies show that EMF affects the formation and differentiation of neural stem cells in the embryological development stage and that prenatal EMF exposure can affect neurological health (Kaplan et al. 2016).

The CN is the first central nervous system nucleus where auditory information is processed, and the cells of the second auditory neurons are located. Animal studies with radiographic marking have shown that neurogenesis

**Table 2** Cleaved caspase-3 positivity score of all groups

| Groups | Neuronal cleaved caspase-3 positivity score (median ± standard deviation) | Oligodendrocytes cleaved caspase-3 positivity score (median ± standard deviation) |
|---|---|---|
| P7 control | $0.00 \pm 0.52^{c,e,g,j,l,n,r}$ | $0.00 \pm 0.41^{c,e,g,j,l,n,r}$ |
| P10 control | $0.00 \pm 0.52^{a,e,g,j,l,n,r}$ | $0.00 \pm 0.52^{a,e,g,j,l,n,r}$ |
| P15 control | $0.00 \pm 0.52^{a,b,g,j,l,n,r}$ | $0.00 \pm 0.41^{a,b,g,j,l,n,r}$ |
| P30 control | $0.00 \pm 0.63^{a,b,e,j,l,n,r}$ | $0.00 \pm 0.41^{a,b,e,j,l,n,r}$ |
| P7 EMF | $2.00 \pm 0.41^{b,f,h,k,m,p}$ | $2.00 \pm 0.52^{b,f,h,k,m,p}$ |
| P10 EMF | $2.00 \pm 0.52^{b,d,f,h,i,m,p}$ | $2 \pm 0.41^{b,d,f,h,i,m,p}$ |
| P15 EMF | $2.00 \pm 0.75^{b,d,f,h,i,k,p}$ | $2.00 \pm 0.52^{b,d,f,h,i,k,p}$ |
| P30 EMF | $4.00 \pm 0.52^{b,d,f,h,i,k,m}$ | $4.00 \pm 0.52^{b,d,f,h,i,k,m}$ |

[a] $P > 0.05$ compared to the P7 control group
[b] $P < 0.05$ compared to the P7 control group
[c] $P > 0.05$ compared to the P10 control group
[d] $P < 0.05$ compared to the P10 control group
[e] $P > 0.05$ compared to the P15 control group
[f] $P < 0.05$ compared to the P15 control group
[g] $P > 0.05$ compared to the P30 control group
[h] $P < 0.05$ compared to the P30 control group
[i] $P > 0.05$ compared to the P7 EMF group
[j] $P < 0.05$ compared to the P7 EMF group
[k] $P > 0.05$ compared to the P10 EMF group
[l] $P < 0.05$ compared to the P10 EMF group
[m] $P > 0.05$ compared to the P15 EMF group
[n] $P < 0.05$ compared to the P15 EMF group
[p] $P > 0.05$ compared to the P15EMF group
[r] $P < 0.05$ compared to the P15 EMF group, Kuskall-Wallis-Tamhane's T2 test

in CN occurs within a week before birth (Pierce 1967; Altman and Bayer 1980; Martin and Rickets 1981). It has been determined that these first connections, which CN neurons recognized in the prenatal period begin to form in the brain stem, are present on the P0th day (Friauf and Kandler 1990); however, somatic maturation in neurons and the formation of the final connection form in adults only occurs in the second week (10–12 days) after birth (Woolf and Ryan 1984; Blatchley et al. 1987; Moore et al. 1998). In line with this information, CN structures were examined histopathologically on 4 non-consecutive days as indicated in the "Materials and methods" section to monitor CN development.

Histological evaluation showed that the histological structures of the VCN in the control groups on for every test day showed normal appearance. However, prenatal EMF application caused structural changes in neurons and oligodendrocytes in CN sections taken at these time points compared to control groups. Several studies have also shown that EMF exposure can have adverse effects on the hearing system. Çeliker et al. (2017) demonstrated that the application of 2100-MHZ EMF for 30 days caused structural damage in the CN. Similarly, in a previous study, neuronal degeneration was observed in rats exposed to 1800-MHz EMF for 30 days (Özgür et al. 2015). However, different from the others in this study, it was observed that 900-MHZ EMF application during the prenatal period in rats also caused structural damage in the VCN. Various studies have reported that 900-MHz EMF exposure during the prenatal period also causes neuronal losses in the hippocampus and brain tissue (Bas et al. 2009; Erdem Koç et al. 2016).

In addition to histopathology, an increased apoptotic index of cochlear nuclei was observed in EMF groups compared to the control group in this study. It has been reported that 1800-MHz and 2100-MHz EMF increases the apoptotic index (Özgür et al. 2015; Çeliker et al. 2017). Even though prenatal EMF application triggered morphological damage in the VCN, the prolongation of ABR wave delays was found to be statistically insignificant. Celiker et al. showed that 2100-MHz EMF application is not at a level that can lead to hearing loss, although it causes structural damage (Çeliker et al. 2017).

In our study, although the prenatal EMF exposure caused structural damage in the CN, no significant difference was found between the groups in the histochemical and immunohistochemical examinations of CN samples. Since this study is the first to investigate the effect of intrauterine EMF application on CN development, there are no others to compare to in the literature. There are some limitations to our study. In our study, the irreversible pathway of apoptosis was evaluated on the caspase-3 cascade. This requires to be supported by molecular studies like tunnel, intracellular, and mitochondrial calcium determinations. In addition, studies on signaling molecules such as p53 and molecular damage mechanisms can be investigated.

In conclusion, this study shows that 900-MHZ EMF applied for 1 h in the prenatal period causes structural damage to the CN but does not affect CN development and does not affect ABR measurements. In future studies, the effects of EMF exposure at different frequencies in the prenatal period will be examined in more detail, and investigate whether it affects the CN development at different frequencies and different exposure periods.

**Author contribution** Levent Tumkaya and Orhan Bas planed and designed the experiments. Levent Tumkaya, Orhan Bas, Tolga Mercantepe, Seda Cinar, and Abdulkadir Ozgur conducted the experiments. Levent Tumkaya, Orhan Bas, Tolga Mercantepe, Seda Cinar, and Zihni Acar Yazici contributed to data collection and interpretation. Levent Tumkaya, Orhan Bas, Tolga Mercantepe, Seda Cinar, Abdulkadir Ozgur, and Zihni Acar Yazici contributed to manuscript preparation.

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**Data availability** The datasets used and/or analyzed during the current study are available from the first author on request (Levent Tumkaya).

## Declarations

**Ethics approval** The study protocol was prepared according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the necessary permissions were obtained from the Local Ethics Committee of Animal Experiments of the Recep Tayyip Erdogan University Faculty of Medicine (no: 37/2010).

**Consent to participate** All the authors equally participated and gave their consent to publish the study in this journal.

**Consent for publication** All the authors gave their consent to publish the study in this journal.

**Conflict of interest** The authors declare no competing interests.

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